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Mechanisms for the myocardial dysfunction observed following hemorrhagic shock remain unclear. A well characterized rabbit model was utilized to determine the effects of shock and retransfusion on myocardial β-adrenergic receptors and adenylate cyclase activity. Hemorrhagic shock was produced by rapid blood withdrawal to reduce mean arterial pressure to 35 mmHg in anesthetized rabbits instrumented for comprehensive hemodynamic monitoring. The shock state was maintained for 60 minutes and then the animals were transfused with the autologous warmed shed blood. Ventricular myocardium was analyzed for [^{3}H] dihydroalprenolol binding and adenylate cyclase activity. There was no effect of shock or retransfusion on basal or stimulated adenylate cyclase activity. However, compared to control (67 ± 11 fmol/mg), β-adrenergic receptor density increased in the shock (136 ± 22 fmol/mg) and retransfusion (152 ± 5 fmol/mg) groups, with no change in apparent ligand affinity. These results suggest that changes in β-adrenergic receptor number and/or adenylate cyclase activity are unlikely to account for the myocardial dysfunction observed following hemorrhagic shock.

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EFFECTS OF HEMORRHAGIC SHOCK AND RETRANSFUSION ON MYOCARDIAL BETA-ADRENERGIC RECEPTORS AND ADENYLATE CYCLASE ACTIVITY

FINAL REPORT: JULY 1, 1990 TO DECEMBER 31, 1990

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INTRODUCTION

Following massive blood loss, cardiac output falls and diastolic filling of the coronary arteries becomes insufficient, resulting in global myocardial ischemia and contractile dysfunction. The internalization of β-adrenergic receptors that normally occurs in response to increased levels of circulating catecholamines in the non-ischemic heart is abolished in the acutely ischemic heart. Consequently, the balance of internalization and externalization of β-adrenergic receptors is shifted during ischemia toward an increase in the number of available receptors at the cell surface. At present, it is unclear whether or not these receptors are effectively coupled to adenylate cyclase, and hence are available for the signal transduction mechanisms which presumably would be necessary to maintain physiological responsiveness and normal contractile function. Alterations in the β-receptor expression and coupling to adenylate cyclase activity, which have been noted in other models of ischemia, have not been previously examined during hemorrhagic shock and retransfusion. Therefore, the present study was undertaken evaluate the effects of hemorrhagic shock and retransfusion on myocardial β-adrenergic binding and adenylate cyclase activity. We employed a well-characterized rabbit model of severe hemorrhagic shock and retransfusion developed in our laboratory.

METHODS

Animal Model

Mature male New Zealand white rabbits were anesthetized with pentobarbital sodium (50 mg/kg i.v.) and then supplemented as necessary throughout the experiment. A tracheostomy was performed and mechanical ventilation instituted with room air. The animals were kept on a warming blanket and under heating lamps to maintain a physiologic rectal temperature (recorded continuously). Continuous ECG monitoring was performed throughout the experiment. A femoral cutdown was performed for introduction of catheters into the descending aorta and inferior vena cava via the femoral artery and vein. The chest was opened in the midline and catheters were placed directly into the right atrium, left ventricle, and main pulmonary artery. An ultrasonic flow probe was fitted onto the ascending aorta. These surgical procedures required no more than 35 minutes to complete. This preparation allowed for continuous monitoring of heart rate and pressures in the descending aorta, right atrium, main pulmonary artery, and left ventricle. Ascending aortic flow was monitored continuously and was used to determine stroke volume and cardiac output. The maximum rate of left ventricular pressure development (LV dP/dt) was derived from the left ventricular pressure curve. Systemic vascular resistance was calculated from the pressure and flow data. Arterial blood gas measurements were obtained every 30 minutes.

Following surgery, the animals were allowed to stabilize for 30 minutes. Control hemodynamic parameters and blood gases were

recorded at the end of this 30 minute period. The animals had blood withdrawn (into a warmed reservoir that contained 100 U/kg of heparin) to reduce mean arterial pressure to 35 mmHg initially. Additional blood was not withdrawn during the following 60 minute shock period, and the intrinsic physiologic compensatory mechanisms were allowed to compensate for this acute loss. Following the 60 minute shock period, the animals were then randomly assigned to either SHOCK or RETRANSFUSION groups. The SHOCK group was not retransfused and animals were killed by pentobarbital overdose at the end of the 60 minute shock period. Animals in the RETRANSFUSION group received a warmed autologous transfusion (over 10 minutes) of heparinized shed blood beginning 60 minutes after the initiation of shock. CONTROL animals were instrumented and monitored for 3 hours but were not bled or transfused. Hemodynamic parameters and blood gases were recorded every 30 minutes throughout the monitoring period. Surviving animals were sacrificed by an overdose of pentobarbital. At the end of each acute experiment, the heart was rapidly harvested and processed to prepare crude sarcolemmal membranes for subsequent receptor ligand binding studies or adenylate cyclase assays. Membranes were stored at -70°C until analyzed.

Preparation of Sarcolemmal Membranes

Methods for preparation of sarcolemmal membrane fractions from rabbit myocardium have been adapted from previously published techniques described for canine myocardium (1). Ventricular

myocardium was minced on ice and homogenized using a Polytron tissue homogenizer (3 bursts for 20 seconds each). The homogenization buffer contained 5 mM Tris-HCl, 0.25 M sucrose, and 1 mM $MgCl_2$. Following homogenization, suspensions were diluted with an equal volume of 1 M KCl and stirred on ice for 10 minutes (this precipitates contractile proteins and reduces non-specific binding). Homogenates were filtered through gauze and centrifuged at low speed for 15 minutes (700 x g). The resulting supernatant was centrifuged at 40,000 x g for 30 minutes and the pellet containing crude sarcolemmal membranes was resuspended in 50 mM Tris-HCl, 10 mM $MgCl_2$ and 1 mM ascorbic acid. The final protein concentration was approximately 10 mg/ml. Protein was determined by the Bradford assay using bovine serum albumin as the standard (2).

Determination of β-Adrenergic Receptor Binding.

Sarcolemmal membranes from control, shock, and retransfused rabbits were utilized for determining β-adrenergic receptor binding characteristics. Assays were performed using an incubation buffer containing 50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM ascorbate and 0.01% BSA (total volume 0.20 ml). Equilibrium binding was performed by incubation at room temperature for 30 minutes with varying concentrations of [^{3}H]-dihydroalprenolol (DHA; specific activity=40-60 Ci/mmol). The concentration of [^{3}H]-DHA ranged from 0.3 to 20 nm. Approximately 250 μg protein was used for each sample. Binding was terminated by addition of ice cold buffer and rapid

vacuum filtration over Whatman GF/C filters using an automated Brandel cell harvester. Specific binding was determined by subtracting binding in the presence of 10 μM dl-propranolol from total binding. The binding isotherms were analyzed using LUNDON software using both linear and non-linear methods.

Measurement of Adenylate Cyclase Activity

Sarcolemmal fractions were used to measure basal and stimulated adenylate cyclase activity in each group (control, shock, and retransfusion). Adenylate cyclase activity was assayed according to the method of Salomon (3). Membranes were incubated for 10 minutes at 30°C using approximately 50 μg protein for each assay. The incubation buffer contained 0.5 mM ATP, 2 mM $MgCl_2$, 1 mM EGTA, 5 mM phosphoenolpyruvate, 0.5 mg/ml pyruvate kinase, 0.1 mg/ml BSA, 0.1 mM isobutylmethylxanthine, and trace [^{32}P]ATP in 80 mM HEPES. The total reaction volume was 0.10 ml. The reactions were initiated by addition of sarcolemmal protein and terminated with a stop solution containing 10 mM cAMP (with trace [^{3}H] cAMP), 40 mM ATP, 1% SDS and with heating to 85°C. The double column method (4,5) is used to separate and quantitate the amount of cAMP formed and recovered. This involves sequential chromatography over Dowex and then alumina oxide. Recovery of authentic cAMP averages 80-90%. The amount of cAMP generated was determined by liquid scintillation spectrometry. Results were corrected for recovery for each individual sample. Assays were performed in the presence and absence of various agonists in order to determine basal and

stimulated adenylate cyclase activities.

Statistical Analyses

Comparisons among groups for the hemodynamic data, β-adrenergic receptor binding data, and results for adenylate cyclase activity were compared by analyses of variance. Fisher's LSD test was utilized when the F value from the analysis of variance indicated a significant difference was present. Statistical significance was defined as $p<0.05$.

RESULTS

Figure 1 illustrates results for mean aortic pressure in the 3 groups. Blood pressure in the control group diminished slightly in the first 30 minutes, but then remained stable throughout the 3 hour monitoring. In contrast, following blood withdrawal, blood pressure fell significantly in the shock and retransfusion groups. Following retransfusion, blood pressure returned to control values by the end of the monitoring period. However, arterial pH remained low (Figure 2) in the retransfusion group.

Specific binding of [^{3}H]-DHA is illustrated in Figure 3. Binding isotherms (specific binding only) are presented for each of the 3 groups. Maximal number of binding sites (B_{max}) was 67±11 fmol/mg in the control group. The number of binding sites identified by DHA increased in the shock group (136±22 fmol/mg) and in the retransfusion (152±5) groups. These were significantly different from control ($p<0.05$). However, as evident in Figure 4,

the apparent ligand affinity did not change appreciably, in that the slopes of the Scatchard-Rosenthal plots are similar for each group. The apparent ligand affinity (K_d) was 7.6±2.4 mM in the control group, 6.9±1.7 nM in the shock group, and 7.8±0.2 nM in the retransfusion group.

Results for basal and stimulated adenylate cyclase activity are illustrated in Figure 5. Basal activities do not differ among the 3 groups. In addition, the responses to the non-hydrolyzable analogue of GTP (GppNHp), forskolin, and sodium fluoride were not statistically different. The degree of stimulation at maximal concentrations of GppNHp, forskolin, and sodium fluoride were similar among the 3 groups. As shown in Figures 6 and 7, the sensitivity to stimulation of adenylate cyclase activity by forskolin and GppNHp were not different among the 3 groups.

DISCUSSION

In the early stages of hemorrhagic shock, global cardiac output is decreased because of severe volume loss, but myocardial function is preserved primarily from distributive changes mediated by an intact neurohumoral response (6,7). In prolonged hemorrhagic shock, as diastolic coronary artery flow decreases, substantial primary myocardial dysfunction occurs (8-10). The mechanisms responsible for cardiac failure in severe hemorrhagic shock are unknown, but experimental observations support the concept of a deterioration in sympathetic influences despite increases in local and systemic release of catecholamines (11). The cardiac

stimulatory actions of catecholamines are mediated primarily by β_1-adrenergic receptors located within the myocardial cell membrane. The β_1-adrenergic receptor is linked to adenylate cyclase via a stimulatory guanine-nucleotide binding protein (G_S). Adenylate cyclase generates the second messenger 3',5'-cyclic adenosine monophosphate (cAMP), which mediates myocardial inotropic and lusitropic responses by activating a specific family of protein kinases that phosphorylate selective proteins in the myocytes. This results in increases in the rate and extent of delivery of Ca^{+2} to the contractile proteins during systole (inotropic benefit) and acceleration of the cytosolic removal of Ca^{+2} during diastole (lusitropic benefit).

In experimental systems the response of "normal" myocardium to elevated catecholamine levels is a reduction in the number of cell surface β-adrenergic receptors, a term often referred to as receptor "down regulation"(12). However, previous studies of myocardial ischemia have conclusively demonstrated an acute increase in the number of cell surface β-receptors (13-22). The increase in the population of β-adrenergic receptors is probably due to a redistribution of receptors from intracellular compartments (so-called internalized receptors) to the sarcolemmal membrane (23,24), and not from rapid synthesis of new receptors. Whether or not this increase in β-receptor density results in increased physiological responsiveness to catecholamines would, however, depend upon the ability to effectively couple the other components of the β-adrenergic transduction system. Data

concerning the effectiveness of this coupling process remain inconclusive (14).

Although the β-receptor density is increased in models of prolonged myocardial ischemia (≥ 1 hour), most of the studies show a decrease in adenylate cyclase activity, suggesting a functional uncoupling of the β-adrenergic transduction system (13-22). This finding has been attributed to a reduced affinity of β-adrenergic receptors for agonists, preventing the formation of a high-affinity ternary complex between agonist, receptor, and stimulatory guanine-nucleotide binding protein (G_s) (12). In support of this concept, Susanni *et al* found a decrease in basal activity and functional responsiveness of the stimulatory guanine-nucleotide binding protein G_s following 1 hour of coronary artery occlusion in a conscious canine model (25). Devos *et al* noted that the density of β-adrenergic receptors increased 35% in canine myocardial membranes 5 hours after ischemic insult, while the proportion of receptors in the high-affinity state (±-isoproterenol) decreased from 43% to 20% (21). Basal and stimulated (NaF, forskolin, GppNHp, isoproterenol) adenylate cyclase activities were all markedly and similarly reduced (21). Freissmuth *et al* also noted a reduction in the proportion of β-adrenergic receptors stabilized in the high-affinity state following prolonged myocardial ischemia, even though total β-adrenoreceptor density was increased (22).

In contrast to these results, others have reported data at variance with a functional uncoupling of the β-adrenergic transduction system during myocardial ischemia. For example,

Mukherjee *et al* found increased levels of cyclic AMP following one hour coronary artery occlusion in the absence (16) and presence (17) of reperfusion. Maisel *et al* noted an increase in isoproterenol-stimulated adenylate cyclase activity in a similar model (23). Will-Shahab *et al* reported a reversible inhibition of adenylate cyclase activity in an anoxic/ischemic rat model, provided that the myocardial ischemic period did not exceed 20 minutes (18).

Our results indicate that β-adrenergic receptor density is increased in this model of acute hemorrhagic shock, and furthermore, this effect is maintained following retransfusion. However, there is no change in adenylate cyclase activity. Thus, changes in β-receptor number or adenylate cyclase activity are unlikely to account for the myocardial dysfunction observed following hemorrhagic shock. Although these data do not exclude functional uncoupling of the β-adrenergic receptor with adenylate cyclase, results from the present studies provide a framework for subsequent investigations to characterize the coupling of β-receptors to adenylate cyclase in hemorrhagic shock and reperfusion.

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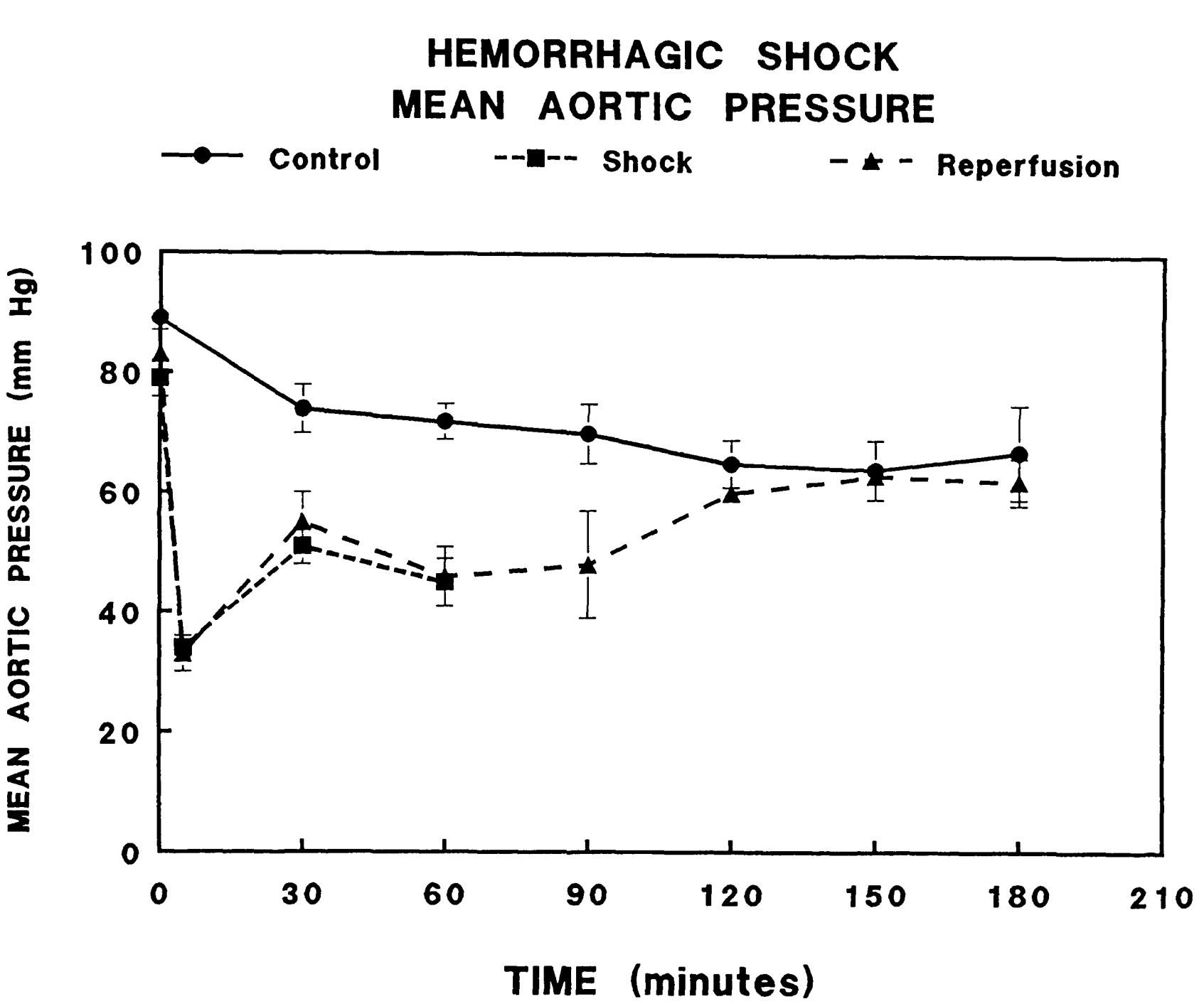
HEMORRHAGIC SHOCK
MEAN AORTIC PRESSURE
Control
Shock
Reperfusion
MEAN AORTIC PRESSURE (mm Hg)
100
80
60
40
20
0
0
30
60
90
120
150
180
210
TIME (minutes)

FIGURE 1

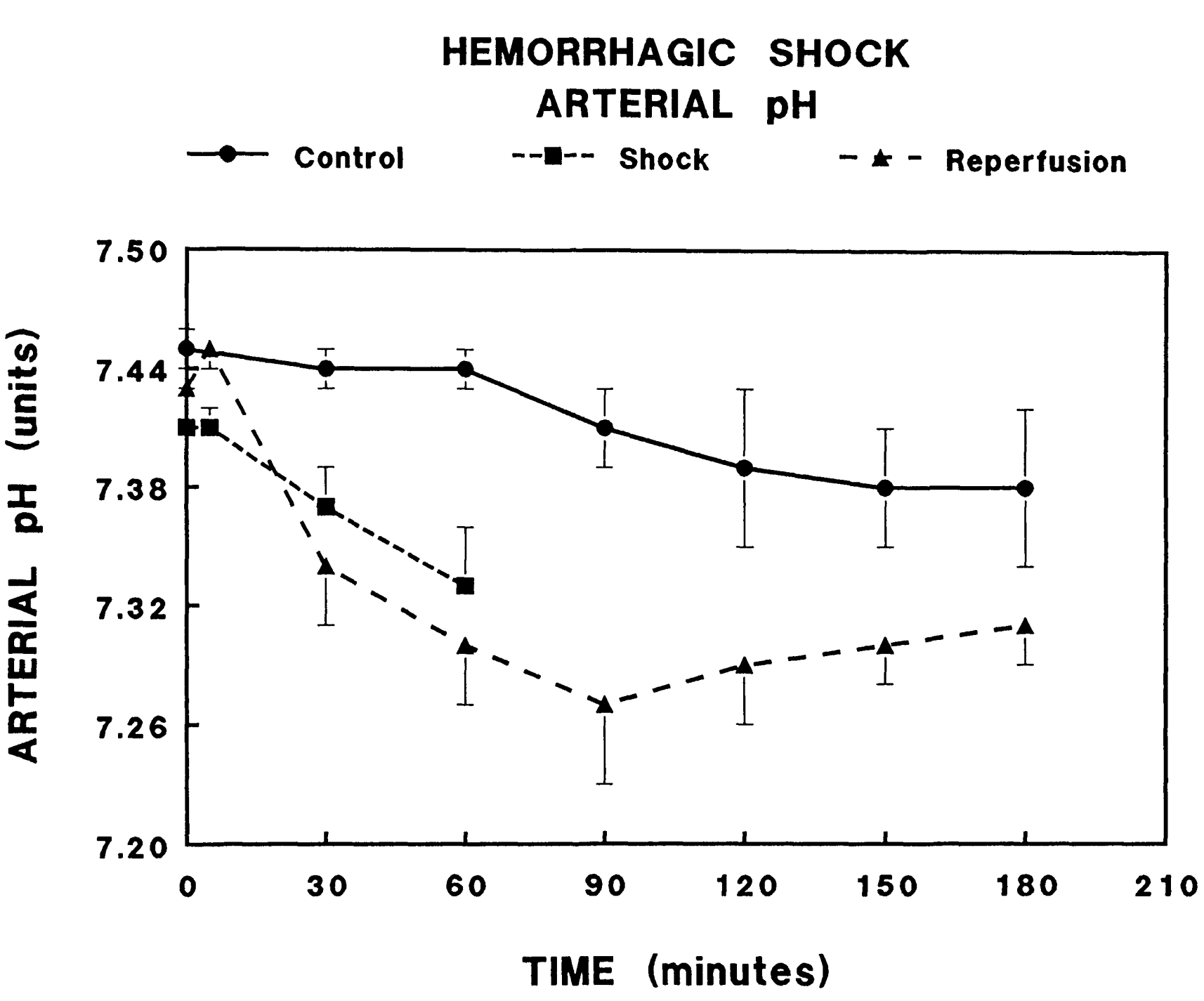
HEMORRHAGIC SHOCK
ARTERIAL pH
Control
Shock
Reperfusion
ARTERIAL pH (units)
7.50
7.44
7.38
7.32
7.26
7.20
0
30
60
90
120
150
180
210
TIME (minutes)

FIGURE 2

BETA-ADRENERGIC RECEPTOR BINDING

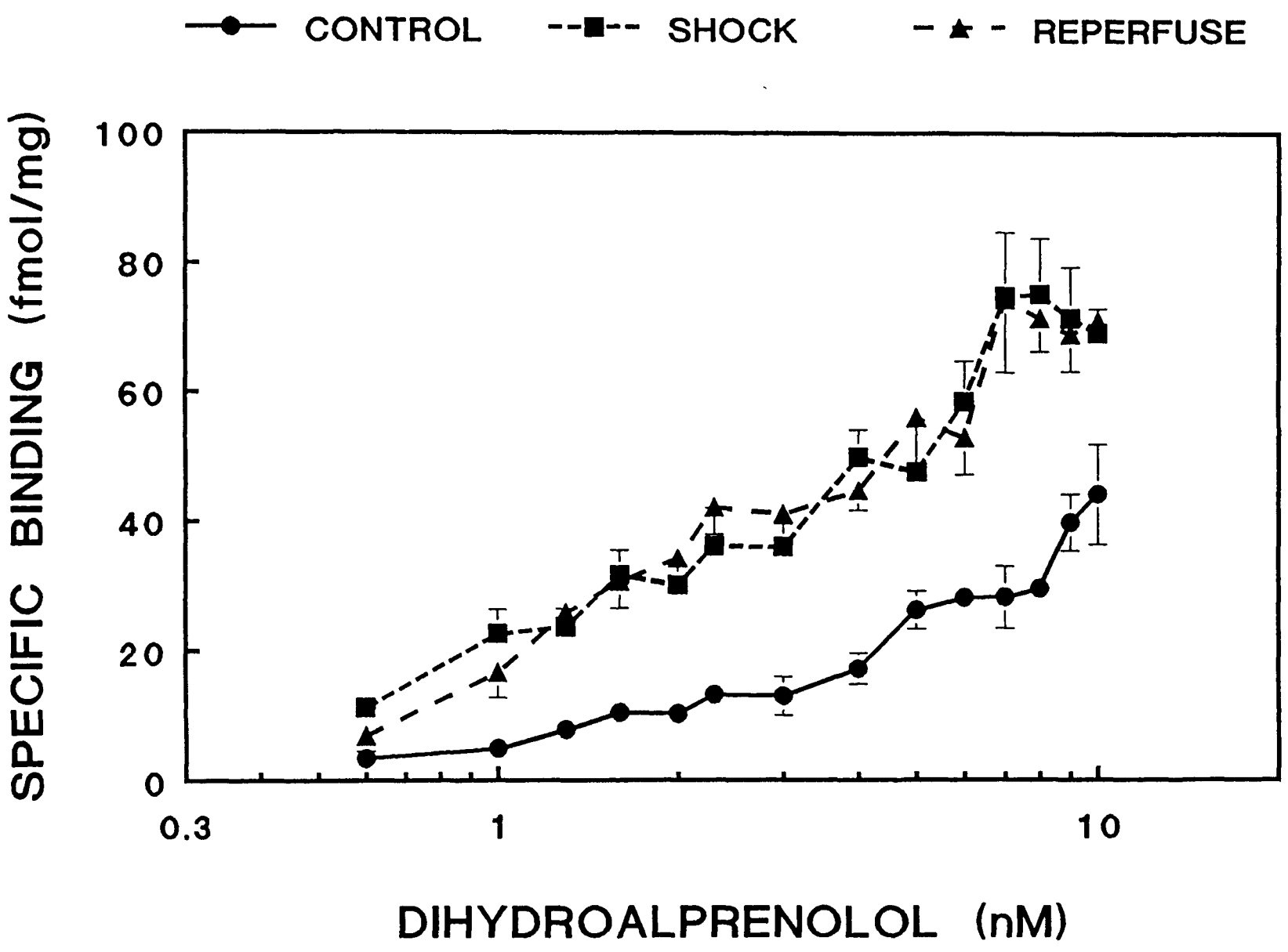

FIGURE 3

SCATCHARD-ROSENTHAL PLOT
DHA BINDING

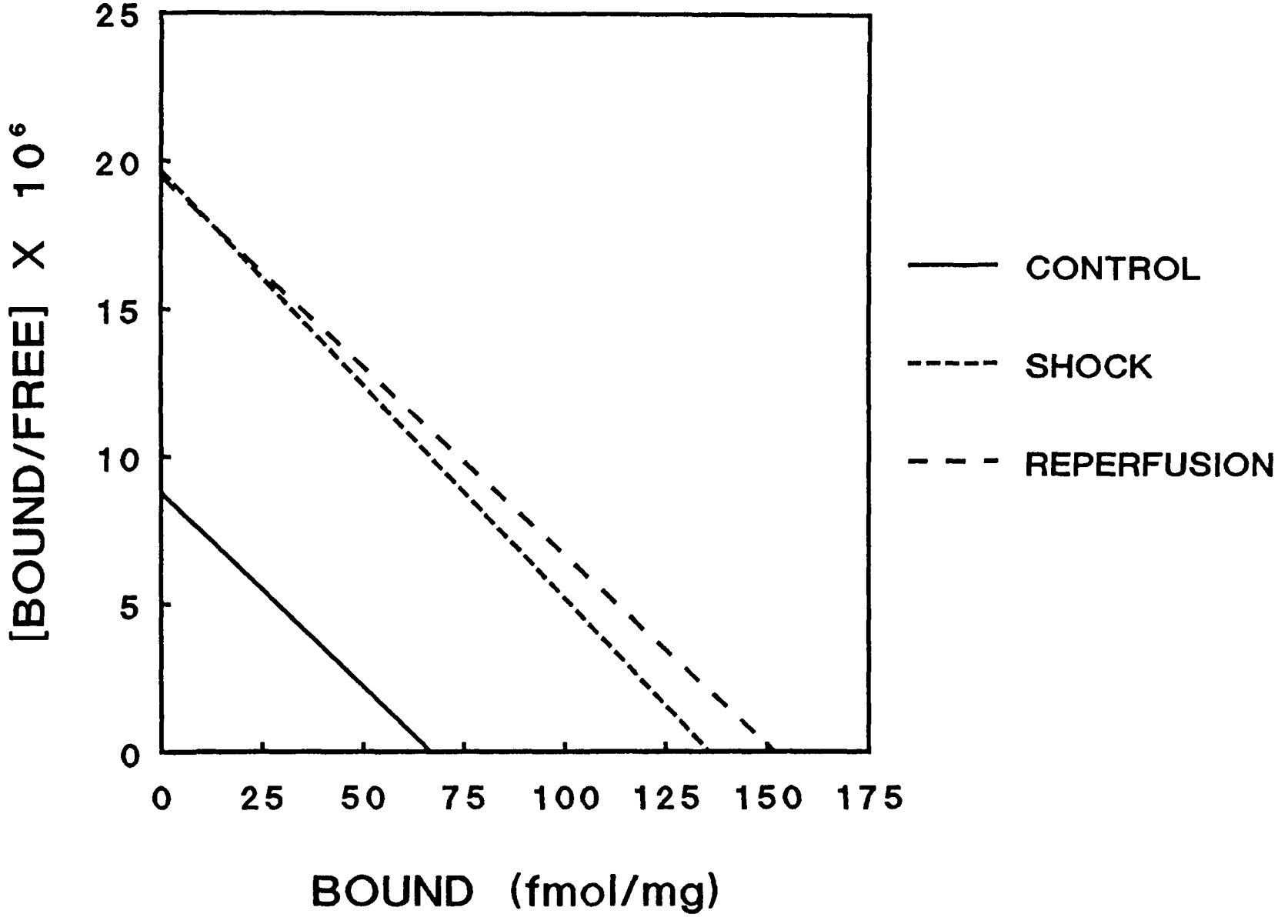

FIGURE 4

ADENYLATE CYCLASE ACTIVITY

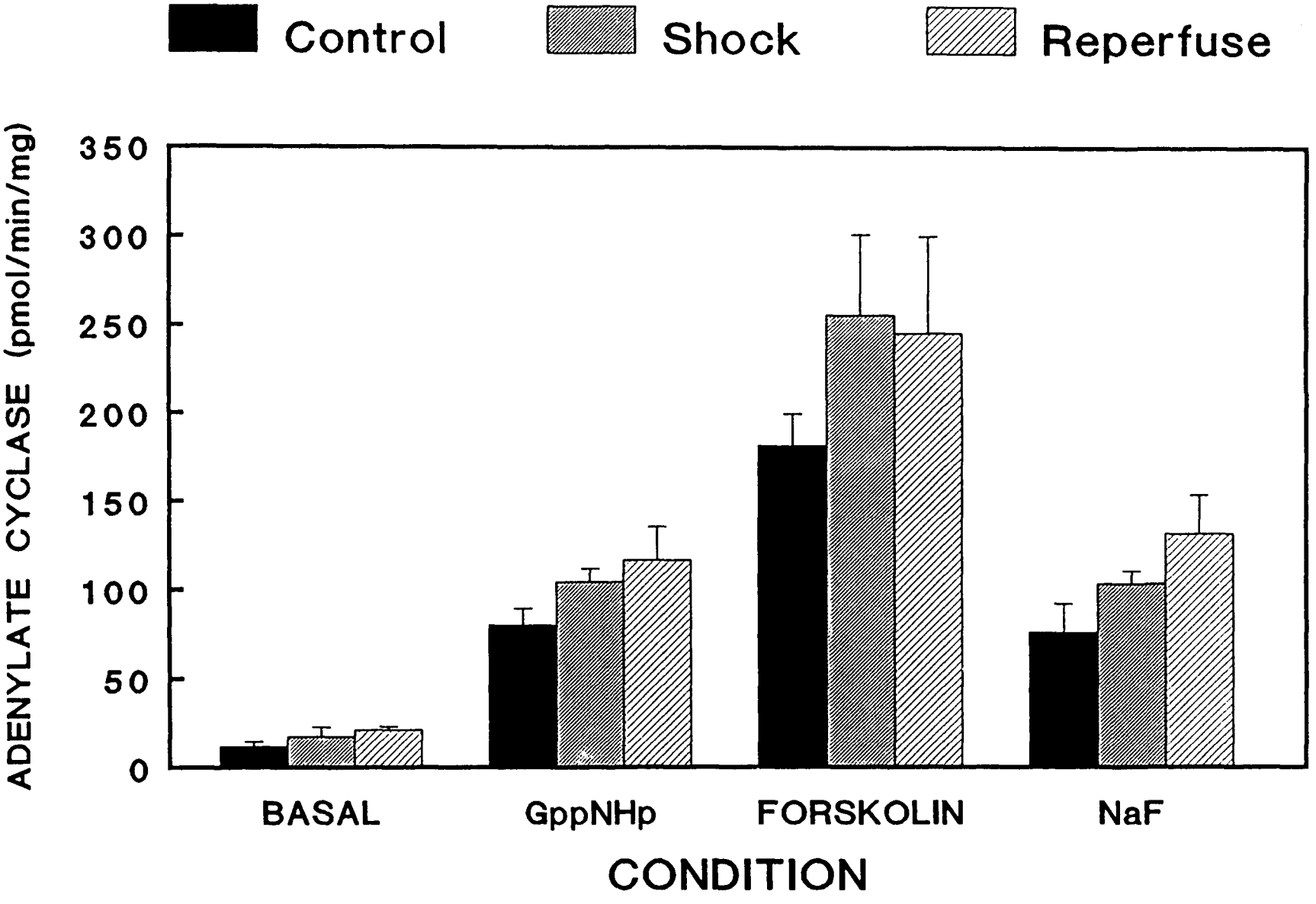

FIGURE 5

ADENYLATE CYCLASE ACTIVITY: EFFECTS OF FORSKOLIN

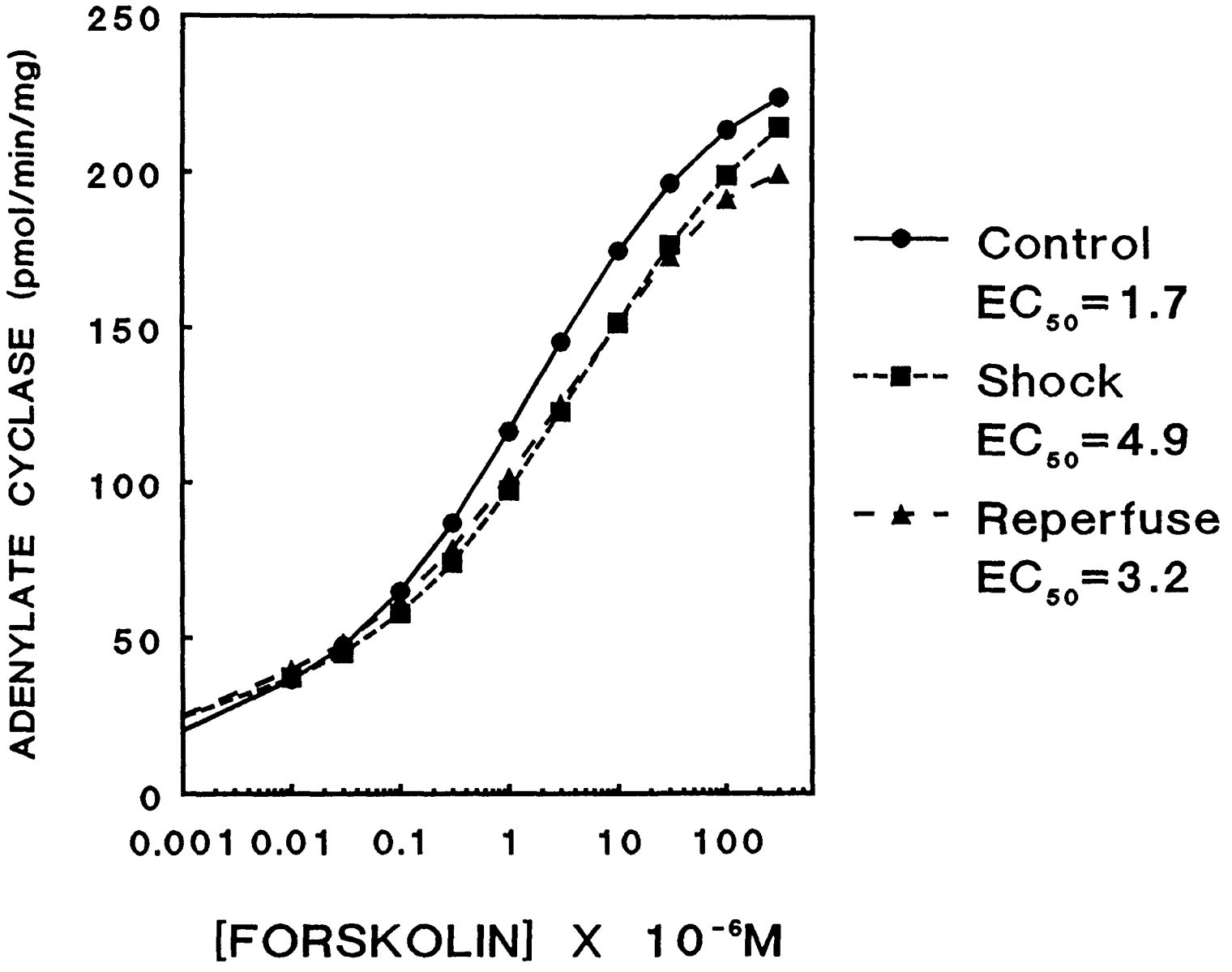

FIGURE 6

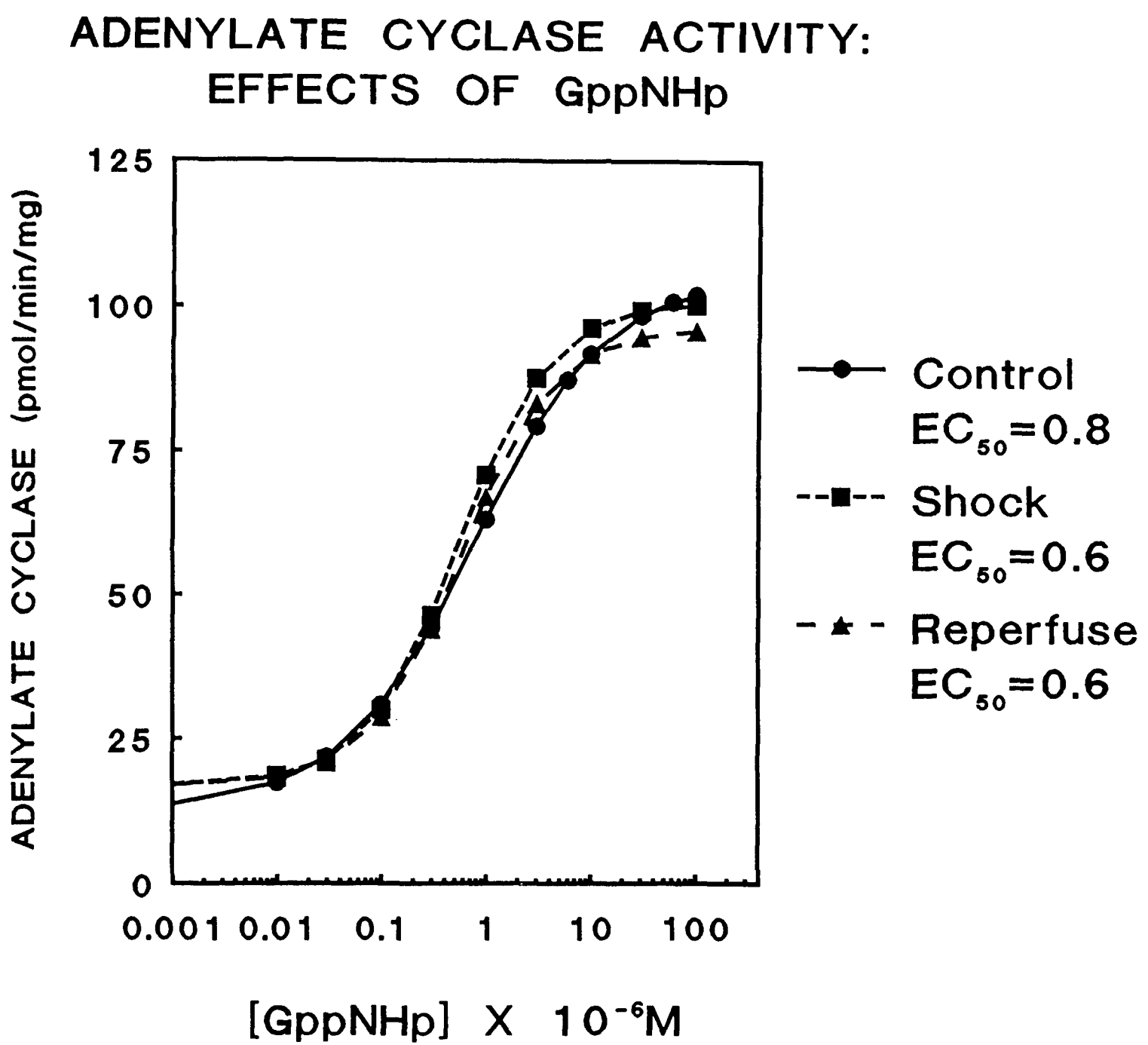
ADENYLATE CYCLASE ACTIVITY:
EFFECTS OF GppNHp
ADENYLATE CYCLASE (pmol/min/mg)
125
100
75
50
25
0
0.001 0.01 0.1 1 10 100
[GppNHp] X 10⁻⁶M
Control
EC50=0.8
Shock
EC50=0.6
Reperfuse
EC50=0.6

FIGURE 7